STAT3 signaling is activated in human skeletal muscle following acute resistance exercise

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Trenerry MK, Carey KA, Ward AC, Cameron-Smith D. STAT3 signaling is activated in human skeletal muscle following acute resistance exercise. J Appl Physiol 102: 1483–1489, 2007. First published January 4, 2007; doi:10.1152/japplphysiol.01147.2006.—The transcription factor signal transducer and activator of transcription 3 (STAT3) has been identified as a mediator of cytokine signaling and implicated in hypertrophy; however, the importance of this pathway following resistance exercise in human skeletal muscle has not been investigated. In the present study, the phosphorylation and nuclear localization of STAT3, together with STAT3-regulated genes, were measured in the early recovery period following intense resistance exercise. Muscle biopsy samples from healthy subjects (7 males, 23.0 ± 0.9 yr) were harvested before and again at 2, 4, and 24 h into recovery following a single bout of maximal leg extension exercise (3 sets, 12 repetitions). Rapid and transient activation of phosphorylated (tyrosine 705) STAT3 was observed at 2 h postexercise. STAT3 phosphorylation paralleled the transient localization of STAT3 to the nucleus, which also peaked at 2 h postexercise. Downstream transcriptional events regulated by STAT3 activation peaked at 2 h postexercise, including early responsive genes c-fos (800-fold), JUNB (38-fold), and c-MYC (140-fold) at 2 h postexercise. A delayed peak in VEGF (4-fold) was measured 4 h postexercise. Finally, genes associated with modulating STAT3 signaling were also increased following exercise, including the negative regulator SOCS3 (60-fold). Thus, following a single bout of intense resistance exercise, a rapid phosphorylation and nuclear translocation of STAT3 are evident in human skeletal muscle. These data suggest that STAT3 signaling is an important common element and may contribute to the remodeling and adaptation of skeletal muscle following resistance exercise.

signal transducer and activator of transcription-3; inflammation; regeneration; hypertrophy

INTENSE MUSCULAR ACTIVITY, particularly eccentric muscular contractions, results in a rapid and localized muscle inflammatory response (5). In the minutes to hours of recovery, a diverse range of proinflammatory cytokines is released by the damaged muscle fibers, and resident neutrophils are rapidly mobilized and activated (30, 42). Typical of the actions of intense exercise is the marked increase in IL-6 (43), which signals via a heterodimer of IL-6 receptor (IL-6R) and glycoprotein 130 (gp130). gp130 is a common element of signal transduction events for a diverse range of cytokine receptors, including those for IL-11, IL-27, leukemia inhibitory factor (LIF), cardiotropin-1, ciliary neurotrophic factor, and oncostatin M (39), which all signal through a common receptor, gp130 (16). gp130 receptor binding activates the signal transducers and activators of transcription (STATs) family via the Janus family of tyrosine kinases (JAKs) (8, 18). Among the seven members of the STAT family (STAT1–4, 5A, 5B, and 6), STAT3 is activated by a wide spectrum of cytokines that signal via the cytokine type II receptor ligands (including IL-6, IL-10, and prolactin), as well as a diverse range of growth factors [including IGF-I, hepatocyte growth factor, epidermal growth factor, PDGF, and basic fibroblast growth factor (16)], ANG II, and mechanical stretch (Rho A) (20, 32, 41). The heterogeneity of signals that may mediate signaling via STAT3 activation suggests that STAT3 is a common and important element to the regenerative responses initiated in the recovery period after intense physical activity.

Activation and phosphorylation of STAT3 elicits dimerization and translocation to the nucleus where it participates in the regulation of many target genes. Subcellular localization of STAT3 in skeletal muscle has been identified in rats following muscle crush injury (21). However, the activation of the STAT3 signaling pathway in human skeletal muscle in response to a remodeling stimulus such as resistance exercise has not been investigated. Importantly, STAT3 activation may be a significant regulator of the transcriptional control of myofibers in the adaptation and recovery following-resistance exercise. First, within proliferating satellite cells, the resident pluripotent stem cell population located within the muscle bed, STAT3 activation is evident (21, 36). Second, within many cell types, STAT3 is important in cell proliferation, differentiation, and survival by mediating the expression of the cell cycle regulators c-myc, cyclinD1 (23, 27), the antiapoptotic genes Bcl-2 and Bcl-XL (23, 27, 46), intermediate early response genes such as c-fos and junB (33), and the angiogenic factor vascular endothelial growth factor (VEGF) (7). These diverse actions have been described in cardiac myocytes, where STAT3 activation mediates cardiac hypertrophy and protection in response to cardiomyopathy induced by ischemia or drug treatment (24, 25).

Therefore, in the present study we investigated the activation of STAT3 signaling following an acute bout of heavy resistance exercise in healthy male volunteers. It was hypothesized that the STAT3 protein would be phosphorylated during the early recovery period following exercise and that this would be accompanied by the subsequent transcriptional activation of STAT3-responsive genes.

MATERIALS AND METHODS

Subjects. Seven untrained but recreationally active male subjects volunteered to participate in this study. The mean (±SE) age, height,
weight, and body mass index of the subjects were 23.0 ± 0.9 yr, 1.78 ± 0.04 m, 76.7 ± 4.5 kg, and 24.1 ± 0.1 kg/m², respectively. Exclusion criteria included resistance training within the past 6 mo, medications, or a previous history of a diagnosed condition or illness that would endanger the subjects during strenuous resistance exercise. Informed written consent was obtained from each subject before participation in the study and after the nature, purpose, and risks of the study were explained. All experimental procedures were performed in accordance with the Helsinki declaration and were formally approved by the Deakin University Human Research Ethics Committee.

Familiarization. Each subject completed a familiarization session on a Cybex NORM dynamometer (Cybex International) to become familiar with the execution of the exercise. The familiarization session involved assessment of isokinetic maximal voluntary contraction (MVC) during a knee extension-flexion exercise. MVC and peak torque (Nm) were determined at 60°/s over 12 maximal repetitions during concentric and eccentric phases. Subjects were instructed to maximally push up and contract throughout the test. The familiarization session took place at least 7 days before the trial to prevent any residual effects of the strength test.

Experimental design. For the 24 h preceding exercise, and the day of the trial, subjects consumed a standard diet (20% fat, 14% protein, and 66% carbohydrate) and abstained from alcohol, caffeine, tobacco, and additional exercise. On the morning of the trial, subjects presented to the laboratory in the fasted state. Following 30 min of supine resting, a muscle sample was collected from the vastus lateralis under local anesthesia (1% Xylocaine) by percutaneous needle biopsy technique (2) modified to include suction (11). Excised muscle tissue from each biopsy was immediately frozen and stored in liquid nitrogen for later analysis.

Following this, subjects completed an acute bout of concentric and eccentric isokinetic leg extension exercise. All exercise was completed using the dominant limb on a Cybex NORM dynamometer at a constant speed of 60°/s. Subjects completed three sets of 12 repetitions of maximal single-legged knee extension exercise with 2 min of rest between each set. The system provided visual graphical feedback for both the subject and the investigator. Subjects were instructed to push up and contract maximally and were verbally encouraged to do so throughout each set. Further muscle samples were collected from the exercised leg at 2 and 4 h after the exercise. Subjects were provided with lunch (30 min following the 4 h muscle biopsy) and dinner (at 1800 for all subjects) on trial day. The following morning, subjects again reported to the laboratory in a fasted state for the collection of a final muscle sample 24 h postexercise. To minimize the potential for interference, serial biopsy samples were collected at least 2 cm from previous biopsy sites.

Protein extraction and Western blot analysis. Tissue samples were homogenized in lysis buffer (20 mM Tris-HCL, pH 7.4, 0.9% NaCl, 100 mM NaF, 2 mM Na3VO4, 1% Igepal, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 3 mM benzamidine, 1 mM PMSF) using a handheld homogenizer. The homogenate was rotated at 4°C for 1 h, then centrifuged at 13,000 rpm at 4°C for 10 min, and the supernatant was collected. Protein samples (50 μg) were denatured in sample buffer and separated by 8% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane and blocked in 5% (wt/vol) BSA in Tris-buffered saline with 0.1% (vol/vol) Tween 20 (TBST) for 2 h at room temperature. Primary antibodies diluted in blocking buffer were applied and incubated overnight at 4°C. Membranes were subsequently washed with TBST and incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies before being washed again. Proteins were visualized by enhanced chemiluminescence (Western Lightning Chemiluminescence Reagent Plus, Perkin-Elmer, Boston, MA). The density of the bands was quantified using Kodak Imaging software, Kodak ID System. The efficacy of antibody coupling-fluorophores, Alexa Fluor goat anti-mouse 488 (no. A21202) or donkey anti-rabbit 594 (no. A21207) (Molecular Probes). Nuclei were stained by incubating with the DNA binding dye bisbenzimide (Hoechst 33258) (Sigma, St. Louis, MO) for 10 min, followed by extensive washing. Immunostained sections were visualized with an Olympus IX70 fluorescence microscope, and digital images were collected using Spot RT slider camera and Magniphot Software (Olympus). Digitally captured images (>200 magnification) with four fields of view per muscle cross section (30 ± 1 fibers per field of view) were processed using ImageJ software (NIH Image). The total number of type I fibers was 12 ± 0 (per field of view) and type II fibers was 18 ± 2 (per field of view). Muscle nuclei were selected based on bisbenzimide staining (74 ± 4 per field of view).

RNA extraction and RT-PCR. Total cellular RNA was extracted using a modification of the phenol-chloroform extraction and isopropanol precipitation protocol, using the ToTALLY RNA Kit (Ambion, Austin, TX). RNA quality and concentration were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). First-strand cDNA was generated from 0.5 μg total RNA using the AMV RT kit (Promega, Madison, WI). RT-PCR was performed using the GenAmp 7500 sequence detection system (Applied Biosystems, Foster City, CA). PCR was performed in duplicate with reaction volumes of 20 μl, containing SYBR/green 1 (Applied Biosystems), forward and reverse primers, and cDNA template (diluted 1:20). Data were analyzed using a comparative critical threshold (Ct) method where the amount of target normalized to the amount of endogenous control relative to control value is given by 2^(-ΔΔCt) (Applied Biosystems). The efficacy of GAPDH as endogenous controls was examined using the equation 2^-ΔΔCt. No changes in the expression of this gene were observed (data not shown), so it was considered an appropriate endogenous control for this study. Primers were designed using Primer Express software package version 3.0 (Applied Biosystems) from gene sequences obtained from GenBank (see Table 1 for details). Primers were designed spanning intron-exon boundaries to prevent amplification of the target region for any contaminating DNA. Primer sequence specificity was also confirmed using Basic Local Alignment Search Tool (BLAST). A melting point dissociation curve was generated by the PCR instrument for all PCR products to confirm the presence of a single amplified product.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 4.1 (GraphPad Software, San Diego, CA). Means were compared using one-way ANOVA, and any significant differences were analyzed using a Newman-Keuls multiple comparison test. Data are presented as means ± SE. A probability level of P < 0.05
RESULTS

STAT3 is phosphorylated in skeletal muscle following acute resistance exercise. To examine STAT3 activation in skeletal muscle, STAT3 phosphorylation (pSTAT3) at tyrosine 705 was examined in vastus lateralis samples from human subjects following a single bout of resistance exercise. At rest, pSTAT3 was undetectable in skeletal muscle (Fig. 1A). By 2 h postexercise, pSTAT3 had increased significantly before returning to near resting levels by 4 h. To confirm that the increased phosphorylation observed was not due to an increase in STAT3 protein levels, the density of the pSTAT3 band was normalized against total STAT3 protein, which remained constant across the samples.

STAT5 phosphorylation is not detectable in skeletal muscle following exercise. We next sought to determine if STAT3 is preferentially phosphorylated in muscle following exercise by examining the activation of another member of the STAT family, STAT5. Interestingly, while STAT5 protein was expressed in skeletal muscle (Fig. 1B), there was no detectable phosphorylation at any time point following exercise, although it was readily detectable in a positive control sample.

Phosphorylated STAT3 translocates to the nucleus postexercise. Phosphorylated STAT3 is able to form homo- and heterodimers and translocates to the nucleus, where it can activate the expression of various target genes. To further explore STAT3 signaling events in skeletal muscle following exercise, the cellular localization of pSTAT3 protein was examined in muscle tissue sections collected at rest, as well as 2, 4, and 24 h postexercise. Immunohistochemical analysis revealed overwhelming translocation of pSTAT3 from within the muscle fibers to the nucleus 2 h after exercise (Fig. 2B), which again returned to resting levels by 4 h. Double immunostaining for pSTAT3 protein and the myosin heavy chain slow protein isoform showed that cytoplasmic pSTAT3 was preferentially localized to the type 2 (fast) fibers (Fig. 2A).

STAT3-responsive genes are transcriptionally activated following acute exercise. We next sought to determine if the increased activation of STAT3 signaling resulted in a concomitant increase in the expression of potential downstream genes c-FOS, JUNB, VEGF, and c-MYC (Fig. 3). Interestingly, was adopted throughout to determine statistical significance unless otherwise stated.

Table 1. Details of primers used for real-time PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Forward Primer (5′–3′)</th>
<th>Reverse Primer (5′–3′)</th>
</tr>
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<tr>
<td>GAPDH</td>
<td>NM_002046</td>
<td>CATCCATGACAAGTTTGATATG</td>
<td>CGACGGGTTTACTGAGGTA</td>
</tr>
<tr>
<td>SOCS3</td>
<td>NM_003955</td>
<td>GACCAATGACATTCTATTG</td>
<td>GCCAGGATGACACTTACAT</td>
</tr>
<tr>
<td>c-FOS</td>
<td>NM_005252</td>
<td>CGCGGCTTGGATGCTCAAG</td>
<td>GCCTCGGTTTTCAAGGTG</td>
</tr>
<tr>
<td>JUNB</td>
<td>NM_002299</td>
<td>GCCGCTAAATGAGACACCTTT</td>
<td>GCCTCGGTTTTCAAGGTG</td>
</tr>
<tr>
<td>VEGF</td>
<td>AY047581</td>
<td>GGCTACATTCTGAGACAGTCT</td>
<td>GCCTCGGTTTTCAAGGTG</td>
</tr>
<tr>
<td>IL-6</td>
<td>NM_000600</td>
<td>GCGAAAGTATGAGATGGAGCT</td>
<td>AGGCCAGCGCAAGATGAAA</td>
</tr>
<tr>
<td>IL-6R</td>
<td>NM_000565</td>
<td>CGCGGCTTGGAGTGTGAGC</td>
<td>AGGCCAGCGCAAGATGAAA</td>
</tr>
<tr>
<td>LIF</td>
<td>NM_002309</td>
<td>GACCAATGACATTCTACAT</td>
<td>GCCATAGCTTGTCAGGTG</td>
</tr>
<tr>
<td>c-MYC</td>
<td>NM_002457</td>
<td>GGCTCCCATGAGACACCTAA</td>
<td>TCTTGGGAGAGATGAGT</td>
</tr>
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</table>

Primer sequences were designed using Primer Express version 2.0 software (Applied Biosystems) using sequences accessed through GenBank and checked for specificity using Nucleotide-Nucleotide Blast search. LIF, leukemia inhibitory factor; SOCS3, suppression of cytokine signaling 3; IL-6R, IL-6 receptor; VEGF, vascular endothelial growth factor.
marked increases in expression were observed for all genes investigated, although differences in magnitude and timing were evident. Significant increases in the expression of the early response genes c-FOS and JUNB (800-fold and 38-fold, respectively) were observed at 2 h postexercise. This increased expression appeared to be largely transient, with a marked reduction evident by 4 and 24 h. Similarly, an increase in c-MYC expression peaked at 2 h postexercise (140-fold) before returning toward resting levels at 4 and 24 h. In comparison, VEGF mRNA expression increases were slightly delayed and of a smaller magnitude, with a fourfold increase observed at 4 h post-resistance exercise.

Genes associated with STAT3 signaling are transiently upregulated following acute resistance exercise. Last, we sought to investigate the expression of several genes implicated in the regulation of STAT3 signaling (Fig. 4). IL-6 and LIF, two cytokines that strongly activate STAT3, demonstrated marked transcriptional activation (85-fold and 120-fold, respectively) at 2 h following exercise. However, because of considerable between-subject variability, the change in LIF failed to reach statistical significance. The expression of IL-6R, through which IL-6 acts, was subsequently investigated. Although no significant changes were observed, a trend toward increased expression was evident at 4 h postexercise (8-fold, P = 0.08). Finally, we investigated the expression of suppressor of cytokine signaling 3 (SOCS3), a well-established negative regulator of STAT3 signaling. Interestingly, the expression of this gene was also markedly increased (60-fold) 2 h after exercise.

DISCUSSION

Skeletal muscle remodeling in response to resistance exercise shares common features with many of the signaling events regulating the regenerative processes following muscle injury. Growth factors and cytokines are central mediators of repair and adaptation in skeletal muscle (42, 45), although the exact signaling pathways activated have yet to be fully elucidated. STAT3 signaling has been identified as a possible mediator of skeletal muscle regeneration (21), but the importance of this...
pathway in the adaptive response of human skeletal muscle to resistance exercise has not been investigated.

The results of the present study demonstrate that an acute bout of resistance exercise in young healthy males rapidly and transiently activates STAT3 signaling in human skeletal muscle. Phosphorylation of STAT3 at tyrosine 705 was undetectable in resting muscle but was markedly increased sevenfold within 2 h following a single session of strenuous leg extension exercise. Tyrosine phosphorylation of STAT3 at this site residue is essential for the formation of homo- or heterodimers, nuclear translocation, and subsequent STAT3-dependent increases in the transcriptional activity of responsive genes (9).

Corresponding to the increased phosphorylation was the demonstration of increased nuclear accumulation of the phosphorylated STAT3 to the nucleus (10-fold) at 2 h postexercise. Interestingly, double labeling for myosin heavy chain I isoforms revealed preferential localization of cytoplasmic phosphorylated STAT3 to the fast (type II myosin heavy chain isoform) fibers. It is possible that greater STAT3 protein is resident within fast fibers or that a greater STAT3 activation is present within these fibers. Further analysis is then required to more adequately describe localization and mechanisms of activation of STAT3 following exercise.

Fig. 3. Increased expression of STAT3 target genes following exercise. mRNA for c-FOS (A), JUNB (B), VEGF (C), and c-MYC (D) were examined in the skeletal muscle of young healthy males at rest or following an acute bout of resistance exercise. Values are arbitrary units normalized to the expression levels of the housekeeping gene GAPDH representing the mean ± SE for 7 individuals. Significantly different from resting levels: *P < 0.05, **P < 0.01.

Fig. 4. Altered expression of genes modulating STAT3 signaling following exercise. Expression of mRNA for IL-6 (A), IL-6 receptor (IL-6R; B), leukemia inhibitory factor (LIF; C), and suppressor of cytokine signaling 3 (SOCS3; D) were examined as described in Fig. 3. Significantly different from resting levels: **P < 0.01, ***P < 0.001.
To further examine the significance of STAT3 signaling in skeletal muscle, we also investigated the activation of another member of the STAT family, STAT5. This has been shown to be activated in skeletal muscle cells by growth hormone (12) and insulin (34), but its activation following resistance exercise has not been described. In contrast to STAT3, we observed no detectable level of STAT5 phosphorylation within skeletal muscle following exercise. While it is conceivable that STAT5 activation occurred outside the time points examined, these results more likely indicate differential activation of the STAT3 and STAT5 family members by acute exercise in human subjects.

The full spectrum of STAT3-regulated genes in skeletal muscle has not yet been characterized; however, potential targets of STAT3 have been known to include c-FOS and JUNB (13, 19), VEGF (14, 29), and c-MYC (3), among others. Having established activation and nuclear translocation of STAT3 in skeletal muscle following exercise, we next sought to determine if this activation was concomitant with increased transcription of STAT3 target genes. Of the genes investigated, the expressions of c-FOS, JUNB, and c-MYC were markedly but transiently upregulated within 2 h after the exercise stimulus, while the increase in VEGF expression peaked at 4 h. Increased expressions of members of the fos and jun gene family have previously been observed within minutes following an acute bout of cycling exercise (33). Fos and Jun proteins constitute the transcription factor complex AP-1, which is purported to regulate the transcriptional activity of genes that contribute to muscle adaptation (15). Increased mRNA levels of VEGF, an important regulator of angiogenesis, are evident in skeletal muscle during hypertrophy before increased capillarization is detected (10, 40). Increases in VEGF expression in human muscle following resistance exercise of a similar magnitude to that described in this study have been reported previously (6). c-MYC, a protooncogene, is a key factor regulating cell growth, transformation, and apoptosis induction in many tissues. In cardiac muscle, increased c-MYC mRNA is observed following exercise (17), while STAT3-induced c-MYC has been demonstrated to mediate cell cycle progression in skeletal muscle (22). However, the precise functions of each of these genes in muscle following resistance exercise are currently unknown. Equally, it cannot be categorically assumed that their upregulation following exercise was a direct result of increased STAT3 signaling and not a result of a myriad of other potential signaling pathways.

Last, we investigated the expression of several genes that positively or negatively regulate STAT3 signaling. While STAT3 is activated by many different ligands, the most widely studied activators in skeletal muscle are IL-6 and LIF. Both IL-6 and LIF mRNA increased markedly within 2 h of the completion of exercise although this increase was transient with levels having returned to their resting state by 4 h. It is generally well established that LIF and IL-6 are released from damaged and exercised muscle (31, 36, 38). LIF has also been established as an important mediator of skeletal muscle hypertrophy in response to increased loading (35). Activation of STAT3 signaling by IL-6 and LIF may contribute to the regeneration and remodeling of skeletal muscle. Conversely, we also demonstrated a marked upregulation in the expression of SOCS3, a negative regulator of STAT signaling, which draws a parallel with previous studies in rat skeletal muscle (37). The SOCS3 gene is itself thought to be a direct target of STAT signaling (4, 28), suggesting that its transcriptional regulation forms part of a classical negative-feedback loop. Such tight control over the magnitude and length of STAT3 signaling is likely to have marked impacts on the physiological outcomes achieved. While the ultimate outcome of STAT3 signaling in this context is not clear, it is intriguing to speculate a role for this signaling pathway in the repair or remodeling of muscle following resistance exercise. Further support for this hypothesis is provided in cardiac tissue where STAT3 signaling may be critical for the development of cardiac hypertrophy (1, 44). Importantly, ongoing research is necessary to fully elucidate the intricacies of the STAT3 signaling pathway and its role in skeletal muscle remodeling.

Collectively, the results of this study describe the activation of the STAT3 signaling pathway in human skeletal muscle following an acute bout of resistance exercise. Although the significance of this signaling in skeletal muscle remains to be determined, the present study suggests that STAT3 signaling may contribute to the remodeling and adaptation of skeletal muscle following resistance exercise.

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